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Effects of long-chain acyl carnitine on membrane fluidity of human erythrocytes

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Amphiphilic compounds such as long-chain acyl carnitines accumulate in ischemic myocardium and potentially contribute to the myocardial damage. To characterize alterations in membrane molecular dynamics produced by palmitoylcarnitine, human erythrocytes were spin-labeled with 5-doxylstearic acid, and membrane fluidity was quantified by measuring the changes in the order parameter derived from ESR spectra. Palmitoylcarnitine induced triphasic alterations in membrane fluidity of human erythrocytes. The membrane fluidity increased for 5 min, then decreased in a concentration-dependent manner. At higher concentrations (100 and 150 μM) of palmitoylcarnitine, membrane fluidity increased again after 30 and 20 min of the incubation, respectively. Addition of 2.8 mM $CaCl_2$ resulted in a significant decrease in membrane fluidity and enhanced the alterations in membrane fluidity caused by palmitoylcarnitine. The results suggest that alterations in molecular dynamics are one mechanism through which long-chain acyl carnitine could play an important role in ischemic injury.

Introduction

Long-chain acyl carnitine has been shown to accumulate in ischemic myocardium due to the inhibition of β-oxidation [1]. The amphiphilic compounds, such as long-chain acyl carnitine and lysophosphoglyceride, have been suggested to be an important factor in ischemic myocardial injury [2,3]. We have already reported that palmitoylcarnitine inhibited the Na^+/K^+-ATPase and adenylate cyclase activity of canine sarcolemma [4], and that it exerted various electrophysiological alterations [5]. These changes could result from the disorganization of membrane composition caused by the detergent action of palmitoylcarnitine. In ischemic myocardium, long-chain acyl carnitine could inhibit plasma membrane Na^+/K^+-ATPase [6], causing an increase in calcium influx through the Na^+–Ca^{2+} exchange mechanism [7]. Calcium has been recognized as the key ion in regulating membrane functions and compositions [8,9]. In this study, we used a spin-labeling technique to obtain information about the molecular mechanism of biomembranes [10,11]. The changes in the order parameter of a spin-labeled fatty acid were measured to evaluate membrane fluidity. Since alterations in membrane fluidity could modulate membrane functions such as ion permeability and enzymatic activities [12,13], we studied the alterations in membrane fluidity of human erythrocytes incubated with palmitoylcarnitine, and the effects of simultaneous application of Ca^{2+} with palmitoylcarnitine on membrane fluidity using a spin-labeling method.

Materials and Methods

Fresh human blood samples from healthy adult donors, collected in heparinized tubes, were centrifuged at 3000 × *g* for 5 min, and the plasma and buffy coat were removed. Erythrocytes were washed three times with cold iso-osmotic NaCl, and then suspended in Hanks' balanced salt solution of the following composition (in mM): NaCl 137/KCl 5.4/glucose 5.6/KH_2PO_4 0.44/Na_2HPO_4 0.33.

Spin-labeling

A fatty acid spin-label, 5-DSA, which has a stable nitroxide radical ring at the C-5 position (counted from the carboxyl group of the acyl chain) was used. Erythrocytes were labeled by incubating 500 μl of a

Abbreviation: 5-DSA, 5-doxylstearic acid.

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50% (v/v) suspension of washed erythrocytes with 1.5 ml Hanks' solution containing 10 μg 5-DSA for 2 h at 4°C. Spin-labeled erythrocytes were washed twice with Hanks' solution to remove unincorporated spin-labels.

Spin-labeled erythrocytes (final hematocrit 12.5%) were incubated with various concentrations of palmitoylcarnitine (30, 60, 100 and 150 μM) and $CaCl_2$ (1 and 2.8 mM) for 5, 10, 20, 30, 45, 60 and 90 min at 37°C in a shaking thermostat bath. $CaCl_2$ (2.8 mM) and palmitoylcarnitine (100 μM) were added simultaneously to spin-labeled erythrocytes, and these mixtures were incubated again. After the incubation, the erythrocytes were suspended in 10 vols of Hanks' solution at 4°C and centrifugated at 3000 × g for 3 min, and the pellet was transferred to a Pyrex capillary tube.

ESR spectrometry

Spectra were obtained with a JES-FE2XG spin-resonance spectrometer (JEOL, Japan) operating at a center field strength of 3280 G with an 8 min scan-time to scan 100 G, a 0.1 s time-constant, a modulation amplitude of 2.0 G and 8 mW of microwave power. Spin-labeled erythrocytes aspirated into a Pyrex capillary tube were placed in a quartz holder, which was maintained at a constant temperature of 37°C.

Reagents

5-DSA spin-label was purchased from Sigma. Palmitoylcarnitine was a gift from Earth Chemical, Japan.

Statistics

All order parameters measured are reported as mean ± S.D. S.D. values were determined using paired or non-paired Student's t-test depending on appropriateness. P values of less than 0.05 were considered significant.

Results

The fluidity of the membrane-incorporated label was quantified by measuring the order parameter, as described by Gaffney [14]. The ESR spectra of human erythrocytes labeled with 5-DSA revealed rapid, anisotropic motion typical of a fatty acid residing within a phospholipid bilayer (see Fig. 1). The order parameter, S, gives a measure of the degree of structural order in the membrane; the order parameter, S, equals 1 for a spin-label moving rapidly about only one axis, and S equals 0 for rapid, isotropic motion. An increase in S is interpreted as a decrease in fluidity.

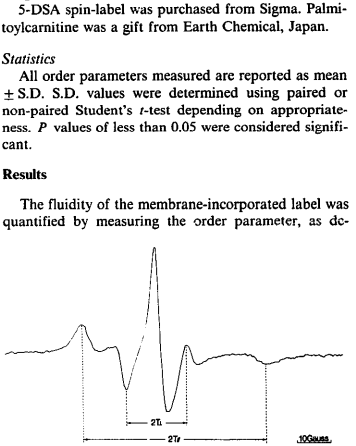

Fig. 1. ESR spectrum of human erythrocytes labeled with 5-DSA were recorded at 37°C. The parallel ($2T_{\parallel}$) and perpendicular ($2T_{\perp}$) components of the hyperfine splittings were measured graphically and the order parameter, S, was calculated from these values.

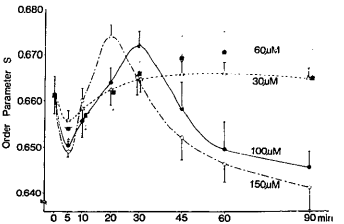

Fig. 2. Effects of palmitoylcarnitine on the order parameter, S. The concentrations of palmitoylcarnitine are the following: △, 30 μM; ■, 60 μM; ●, 100 μM and ○, 150 μM. Values are means ± S.D. (n = 6).

Effects of palmitoylcarnitine on membrane fluidity

The effects of palmitoylcarnitine on membrane fluidity are shown in Fig. 2. The order parameter decreased during the first 5 min (30 μM; 0.6555 ± 0.0020, 60 μM; 0.6540 ± 0.0025, 100 μM; 0.6505 ± 0.0010, 150 μM; 0.6490 ± 0.0015) by the incubation of erythrocytes with palmitoylcarnitine. At 30 μM palmitoylcarnitine, the order parameter increased after 5 min of incubation, and reached a plateau at 30 min. At 60 μM palmitoyl carnitine, the order parameter increased after 5 min and was maximal at 60 min, and then decreased slightly from 0.6705 ± 0.0045 at 60 min to 0.6650 ± 0.0060 at 90 min. At 100 μM palmitoylcarnitine, the order parameter increased after 5 min of incubation and was maximal at

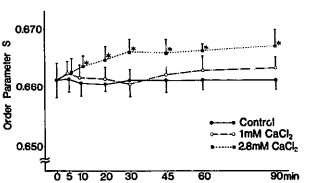

Fig. 3. Effects of $CaCl_2$ on the order parameter, S. The concentrations of $CaCl_2$ are the following: ●, control; ○, 1 mM and ■, 2.8 mM. Values are means ± S.D. (n = 5). * P < 0.05 compared to control.

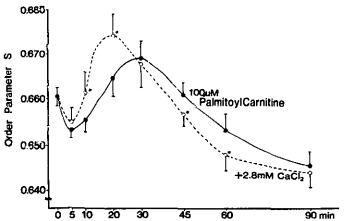

Fig. 4. Effects of simultaneous application of 2.8 mM $CaCl_2$ with 100 μM palmitoylcarnitine on the order parameter, *S*. The erythrocytes were incubated with 100 μM palmitoylcarnitine in the presence (○) or the absence (●) of 2.8 mM $CaCl_2$. Values are means ± S.D. ($n = 5$). * $P < 0.05$.

30 min (0.6720 ± 0.0030), and decreased thereafter. At 150 μM palmitoylcarnitine, the curve was shifted to the left compared with that of 100 μM, and the maximal increase in the order parameter was at 20 min (0.6740 ± 0.0025). After 5 min of incubation, the membrane fluidity of human erythrocytes was reduced by palmitoylcarnitine in a concentration-dependent manner.

Effects of $CaCl_2$

Incubation of erythrocytes with 1 mM $CaCl_2$ did not produce significant alterations in the order parameter of 5-DSA (Fig. 3). Incubation with 2.8 mM $CaCl_2$ resulted in a significant increase in the order parameter after 10 min incubation. Simultaneous application of 2.8 mM $CaCl_2$ with 100 μM palmitoylcarnitine produced a significant increase in the order parameter comparing with that of 100 μM palmitoylcarnitine alone (Fig. 4).

Discussion

This study has demonstrated that the addition of palmitoylcarnitine to human erythrocytes resulted in alterations in membrane molecular dynamics. Palmitoylcarnitine has been shown to accumulate in membrane structure, especially in the sarcolemma of myocytes during myocardial ischemia [15], due to the restrictive oxidative metabolism of free fatty acids. The increased concentration of palmitoylcarnitine during ischemia might effect profound disturbances in membrane function and induce electrophysiological changes [4,5]. However, the mechanism responsible for these changes is not fully understood. We have used human erythrocytes as a membrane model system in this study, since the membranes of erythrocytes are probably the best-understood of all cellular membranes in terms of molecular composition and function [16], are free from oxidation pathway of free fatty acids, and are available without contaminating cells.

Palmitoylcarnitine induced triphasic alterations in membrane fluidity of human erythrocytes. The increase in membrane fluidity during the first phase could be caused by the direct effects resulting from the detergent action of palmitoylcarnitine. Fink and Gross [17] have also demonstrated that amphiphilic compounds, such as lysophosphoglycerides and long-chain acyl carnitines, increased membrane fluidity of canine myocardial sarcolemma after 30 s incubation. The amphiphilic compounds, which contain both hydrophilic and hydrophobic groups, can be incorporated in the lipid bilayer of the membrane [18]. This incorporation could be the primary step which triggers alterations in membrane functions and structure.

The membrane fluidity decreased after 5 min incubation with palmitoylcarnitine. The decrease in membrane fluidity was also observed in the erythrocytes incubated with 2.8 mM $CaCl_2$. It has been shown that addition of calcium decreased membrane fluidity in rat adipocyte ghosts, and small-intestinal brush-border membranes [19,20]. Simultaneous application of $CaCl_2$ with palmitoylcarnitine significantly enhanced the decrease in membrane fluidity during the second phase, but caused no alterations in membrane fluidity during the first phase. Recently, we have demonstrated electrophysiologically that palmitoylcarnitine induced delayed postdepolarization and postcontraction, which indicate intracellular calcium overload [21]. It is likely that the decrease in membrane fluidity may be related to the increase in intracellular calcium concentration.

It has been reported that palmitoylcarnitine enhanced calcium release from the sarcoplasmic reticulum, and inhibited sarcolemmal Na^+/K^+-ATPase [4,6], which could cause an activation of the Na^+–Ca^{2+} exchange mechanism in myocardium [22]. Na^+–Ca^{2+} exchange may play a role in regulating the cellular calcium level in several tissues, e.g., dog erythrocytes, squid axon and cardiac muscle [23,24]. However, McNamara et al. [25] and Sarkadi [26] have demonstrated that a significant Na^+–Ca^{2+} exchange was absent from human erythrocytes with physiological internal sodium concentration. Another possibility of calcium influx is an enhancement of passive calcium transport due to the disorganization of the membrane.

Calcium in micromolar concentrations may alter the configuration of the spectrin-actin network attached to the internal surface of the erythrocyte membrane, and may contribute to the irreversible stiffening of the membrane under certain conditions [27]. Recently, it has been shown that an increase in the cytoplasmic calcium level of the erythrocytes induced marked alterations in the transbilayer organization of the membrane phospholipids accompanied by changes in cell shape, membrane-protein composition, intracellular ATP levels and

transglutaminase activity, and these changes might affect the decrease in membrane fluidity [28].

These results suggest that the decrease in membrane fluidity observed in the second phase could have been caused by alterations in the membrane–lipid interaction and the transbilayer disorganization of the membrane phospholipids.

In the third phase, membrane fluidity increased again, and we observed these changes coinciding with the onset of hemolysis. Tanaka and Ohnishi [29] have demonstrated that the membrane fluidity of human erythrocytes increased during hemolysis using phosphatidylcholine spin-label. It has been suggested that the increase in membrane fluidity of the third phase may reflect the changes in the distribution of fatty acid spin-labels caused by the degradation of the membrane phospholipids.

This study has demonstrated that palmitoylcarnitine induces alterations in the membrane fluidity of human erythrocytes, and that simultaneous application of calcium and palmitoylcarnitine enhances the alterations in membrane fluidity. Since it has been shown that both long-chain acyl carnitine and calcium accumulate in the ischemic myocardium, these results support the hypothesis that alterations of the biophysical properties of membranes produced by amphiphiles play an important role in myocardial ischemia, and that the accumulation of calcium amplifies the damage to membranes weakened by amphiphiles.

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